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Optimized protein extraction methods for proteomic analysis of *Rhizoctonia solani*

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Abstract: *Rhizoctonia solani* (Teleomorph: *Thanatephorus cucumeris*, *T. praticola*) is a basidiomycetous fungus and a major cause of root diseases of economically important plants. Various isolates of this fungus are also beneficially associated with orchids, may serve as biocontrol agents or remain as saprophytes with roles in decaying and recycling of soil organic matter. *R. solani* displays several hyphal anastomosis groups (AG) with distinct host and pathogenic specializations. Even though there are reports on the physiological and histological basis of Rhizoctonia-host interactions, very little is known about the molecular biology and control of gene expression early during infection by this pathogen. Proteomic technologies are powerful tools for examining alterations in protein profiles. To aid studies on its biology and host pathogen interactions, a two-dimensional (2-D) gel-based global proteomic study has been initiated. To develop an optimized protein extraction protocol for *R. solani*, we compared two previously reported protein extraction protocols for 2-D gel analysis of *R. solani* (AG-4) isolate Rs23. Both TCA-acetone precipitation and phosphate solubilization before TCA-acetone precipitation worked well for *R. solani* protein extraction, although selective enrichment of some proteins was noted with either method. About 450 spots could be detected with the densitiometric tracing of Coomassie blue-stained 2-D PAGE gels covering pH 4–7 and 6.5–205 kDa. Selected protein spots were subjected to mass spectrometric analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Eleven protein spots were positively identified based on peptide mass fingerprinting match with fungal proteins in public databases with the Mascot search engine. These results testify to the suitability of the two optimized protein extraction protocols for 2-D proteomic studies of *R. solani*.

Key words: MALDI-TOF-MS, proteomics, *Rhizoctonia solani*, *Thanatephorus cucumeris*, 2-D PAGE

INTRODUCTION

About 90% of the 2000 major diseases of principle crops in the United States are caused by soilborne plant pathogens (Lewis and Papavizas 1991) resulting in losses in excess of $4 billion/y (Lumsden et al 1995). The basidiomycetous soilborne fungus *Rhizoctonia solani*, (Tele: *Thanatephorus cucumeris*, *T. praticola*) is known to attack 188 species of higher plants in 32 families, including various staple crops, ornamentals and turf grasses (Manibhushanrao et al 1981, Anderson 1982). In addition to important plant pathogens of agricultural crops, it includes isolates mutualistically associated with orchids and mosses and saprophytes responsible for recycling soil organic matter (Cubeta and Vilgalys 2001). There are at least 13 hyphal anastomosis groups (AG) of *R. solani* (Carling et al 2002). AGs are considered genetically isolated groups with distinct host specializations, epidemiology and sensitivity to pesticides. Isolates belonging to AG-4 have wide host range and most often are pathogenically associated with ornamental plants.

The physiological and histological basis of host-pathogen interactions in both pathogenic and non-pathogenic Rhizoctonia-host combinations has been documented (Dodman and Flintje 1970, Akino and Ogoshi 1995, Keijer 1996, Weinhold and Sinclair 1996). AG-4 and AG-5 isolates respectively form infection cushions and lobate appressoria (Akino and Ogoshi 1995). The infection process is thought to be both mechanical and enzymatic; the enzymes involved being α-amylase, cellulase, chitinase, pectinase, pectin lyase, β-glucanase, protease and laccase (Bertagnolli et al 1996, Bora et al 2005). However the correlation between pectolytic and cellulolytic en-

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zymes with pathogenicity of *R. solani* is not clear (Akino and Ogoshi 1995).

In spite of detailed physiological and histpathological investigations, there is a huge gap in our knowledge about the molecular biology and control of gene expression during *R. solani* infection (Akino and Ogoshi 1995). Exploration of the Rhizoctonia genome and its regulation at the gene and protein levels should not give only a better understanding of the biological and ecological adaptation of the pathogen, but it also will add to the plant pathologist's arsenal potentially diverse and novel control strategies based on modern molecular approaches. We reported recently the characterization of two distinct dsRNA genomes associated with virulence and hypovirulence of the pathogen (Jia et al 1997, 1998, Lakshman et al 1998). In addition we reported the regulation of *R. solani arom* gene in virulence as well as in chemically and dsRNA-induced hypovirulence of the fungus (Lakshman et al 2006). So far no investigation on the molecular mechanism of *R. solani* pathogenesis at the global proteomics level has been reported.

Proteomic technologies are powerful tools for examining alterations in protein profiles (Gorg et al 2000, Dubey and Grover 2001). It involves the combined applications of advanced separation techniques such as 2-D gel electrophoresis, identification techniques such as mass spectrometry analysis and bioinformatics tools to characterize the proteins in complex biological mixtures. Proteomic profiling by 2-D gel analysis for qualitative as well as quantitative global gene expression studies and its superiority over targeted gene analysis alone has been established (Lehesranta et al 2005, Ruebelt et al 2006). It also is desirable to study proteomics in addition to analyzing mRNA expression because highly expressed mRNAs do not necessarily correlate with protein expression and turn-over (Gygi et al 1999, Tsai-Morris et al 2004) and genes required for a response are not necessarily the same genes that are differentially regulated as a result of the response (Birrel et al 2002, Giaever et al 2002, Thurston et al 2005). Also spliced variants of genes are known to code altogether different types of proteins (Blencowe and Khanna 2007, Cheah et al 2007) and posttranslational modifications of a single protein may lead to multiple functions (Davison 2002, Rodríguez-Piñeiro et al 2007). Besides proteomics allow location-specific analysis (i.e. subproteomes at the level of organelles, cell membranes, cell wall, secretory proteins, etc.), the study of post-translational modifications (Kim et al 2007) and interactions of host-pathogen as well as host-pathogen-biocontrol agents (Marra et al 2006, Rampitsch et al 2006).

Filamentous fungi have an exceptionally strong cell wall and cell lysis as well as protein recovery remained the most difficult steps in proteomic studies (Nandakumar and Marten 2002, Shimizu and Wariishi 2005). Although methods for 2-D protein analysis of limited filamentous plant pathogenic fungal species have been published (Fernández-Acero et al 2006, Rampitsch et al 2006, Kim et al 2007), procedures for protein extraction and 2-D gel analysis conditions are progressively evolving based on individual characteristics of fungal pathogens. Mara et al (2006) reported in a limited fashion the 2-D gel separation of total proteins from an isolate of *R. solani* pathogenic to tomato. Of note, tomato could be infected by several AGs of both multinucleate and binucleate Rhizoctonia representing at least two genera (Sneh et al 1991, Elbakali et al 2003, Balai and Kowsari 2004, Gao et al 2006). However the AG status of the isolate used in the study was not reported by Mara et al (2006). The authors also were unable to molecularly identify any protein from a limited number of MALDI-TOF-MS analyzed spots from 2-D gels by homology in the NCBInr database. To our knowledge our communication is novel for the 2-D gel separation and identification of proteins from a known AG (i.e. AG-4) isolate of *R. solani*.

In this study we modified and evaluated two protein extraction methods originally found to be optimal for an ascomycetous fungus, *B. cinerea*, and for soybean seed proteins respectively (Natarajan et al 2005, Fernández-Acero et al 2006). We observed that both extraction protocols with modifications work well in our investigation. Due to the absence of an *R. solani* protein or DNA database, limited protein identification in this investigation initially was achieved by peptide mass fingerprint matching to cross-species fungal sequences. In addition the relative merits of the two protocols have been evaluated for proteomic studies of *R. solani*.

MATERIALS AND METHODS

Fungal isolate and growth condition.—*R. solani* AG-4, isolate Rs 23 (Lewis and Papavizas 1987) infects ornamental plants. The fungus was grown in potato dextrose broth in 500 mL flasks at room temperature in darkness (Lakshman and Tavantzis 1994). The mycelium was harvested after 7 d, washed four times with autoclaved and de-ionized water, blotted to remove excess water, lyophilized and stored at −20 C until further processing.

Protein extraction protocols.—Total proteins from mycelia of *R. solani* were extracted with two reported protein extraction protocols with modifications. The mycelia grown from the same set of flasks at a particular time point were harvested and split between the two protocols. In the first method (trichloroacetic acid [TCA]-Acetone or TA), originally developed for protein extraction for 2-D gel

analysis of soybean seed proteins (Natarajan et al 2005), 1 g lyophilized mycelium (5.55 g mycelial fresh weight) was powdered in liquid nitrogen with a mortar and pestle and the powder was suspended in 15 mL of 10% (w/v) TCA in acetone containing 0.07% (v/v) 2-mercaptoethanol. The suspension was vortexed and incubated at −20 C for 1 h with intermittent stirring. Total protein was recovered by centrifugation at 14 000 rpm for 20 min at 4 C. The pellet was washed with acetone containing 0.07% (v/v) 2-mercaptoethanol, dried under vacuum for 30 min and resuspended in 1 mL of lysis buffer (9 M urea, 1% 3-[{3-cholamidopropyl} dimethylammonio]-1-propane sulfonate) (CHAPS), 1% (v/v) immobilized pH gradient (IPG) buffer 4–7 or 6–11 (Amersham Biosciences, Piscataway, New Jersey) and 1% dithiothreitol (DTT)) by ultrasonication in an ice-cold water bath. Cell debris was removed by centrifugation at 14 000 rpm for 10 min at 4 C. The extraction was repeated with mycelium grown at two different times.

The second method is based on phosphate solubilization (Evangelista et al 1998, Bora and Ribeiro 2004, Fernández-Acero et al 2006) before TCA-acetone precipitation (phosphate-TCA-acetone or PTA). We have adapted a protocol originally reported to be suitable for protein extraction of an ascomycetous filamentous fungus *B. cinerea* (Fernández-Acero et al 2006). In this method 1 g lyophilized mycelium (5.55 g mycelial fresh weight) was powdered in liquid nitrogen and suspended in 5 mL of 10 mM phosphate buffer (pH 7.4) in the presence of 0.07% w/v DTT and 250 µL/20 mL of a inhibitor cocktail for use with fungal extracts (Catalog No. P8215, Sigma Chemical Co., St Louis, Missouri) under constant stirring on ice for 2 h. The mixture was clarified by centrifugation at 14 000 rpm for 20 min and the phosphate solubilized protein was precipitated with 4 volumes of TCA-acetone and resolubilized according to Natarajan et al (2005). As in TA-method, the PTA-extraction method was repeated with mycelium grown at two different times.

Determination of protein concentration and electrophoresis.—The concentrations of protein extracted by the above two methods were determined by the Bradford method (Bradford 1976) with a protein assay dye kit from Bio-Rad. Aliquots of all protein samples were precipitated with 10% TCA, centrifuged and rinsed with cold acetone. Samples were air-dried and resolubilized in 1N NaOH. Concentration of protein in each sample was calculated against a standard curve of bovine serum albumin.

The first dimension isoelectric focusing (IEF) was performed in an IPGphor system (GE Healthcare, Wauwatosa, Wisconsin) with immobilized pH gradient gels (Immobiline pH 4–7 and pH 6–11, 13 cm, GE Healthcare). IPG strips (pH 4–7 and pH 6–11) containing 500 µg of protein were rehydrated with 250 µL hydration buffer (8 M urea, 2% CHAPS, 0.5% Pharmalyte, 0.002% bromophenol blue and DTT) according to the manufacturer's recommendations. The settings for IEF for pH 4–7 strips were 30 V for 14 h, 500 V for 1 h, 1000 V for 1 h and finally 8000 V untill it reaches 25 k Vh. The voltage for pH 6–11 strips were 30 V for 13 h, 500 V for 1 h, 1000 V for 1 h and 8000 V to a total of 30 000 Vh. The focused strips were run immediately or stored at −80 C in screw-cap glass vials. For second dimension SDS-polyacrylamide gel-electrophoresis (PAGE), the strips were equilibrated with equilibration buffer I (50 mM Tris-HCl pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 1% DTT) and equilibration buffer II (50 mM Tris-HCl pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 2.5% iodoacetamide) for 20 min each on a shaker. The equilibrated strips were placed on 12.5% 2-D gels (18 × 16 cm) with tris-glycine SDS electrophoresis buffer Laemmli (1970). Strips were overlaid with agarose sealing solution. The gels were run on an electrophoresis unit (Hoefer SE 600 Ruby) according to the manufacturer's recommendations, until the tracking dye reached the bottom of the gel (GE Healthcare). After electrophoresis gels were removed from their cassettes and were fixed overnight in 45% ethanol and 10% acetic acid solution. Next morning gels were subjected to 4 × 15 min washes with distilled water. Gels were equilibrated in 34% methanol, 17% ammonium sulfate and 3% phosphoric acid solution for 1 h and finally incubated with 0.66% colloidal Coomassie blue G-250 in equilibration buffer (Newsholme et al 2000) for 2 d at room temperature on an orbital rotator. The gels were washed with distilled water, stored in 20% ammonium sulfate solution and scanned with laser densitometry in a PDSI scanner from GE healthcare. Eight 2-D gels of pH 4–7 were run from samples of two extraction methods and with mycelium grown at two time points. In addition we repeated the two extraction methods with mycelium grown from single time point and run one 2-D gel (pH 4–7) from each extraction. Thus the total number of 2-D gels run was 10. Three representative 2-D gels for each extraction were used to estimate number of spots.

Protein identification.—Selected protein spots (FIGS. 1, 2) were excised from the 2-D PAGE gel, rinsed with distilled water and equilibrated with 50% acetonitrile containing 25 mM ammonium bicarbonate to remove Coomassie blue stain. The gel plugs were dehydrated with 100% acetonitrile, vacuum dried, and digested with 20 µL of 10 µg/mL modified porcine trypsin (sequencing grade, Promega, Madison, Wisconsin) in 25 mM ammonium bicarbonate and incubated overnight at 37 C. The tryptic fragments were eluted from the gel with 50% acetonitrile and 5% trifluoroacetic acid by sonication. The extracts were dried under vacuum and dissolved in 5 µL of 50% acetonitrile and 0.1% trifluoroacetic acid. An aliquot of 0.5 µL from each sample was cocrystallized with α-cyanohydroxycinnamic acid (CHCA) matrix on a multiwell sample plate and air dried before MALDI-TOF-MS analysis.

Tryptic peptides were analyzed with a Voyager DE-STR MALDI-TOF-MS spectrometer (Applied Biosystems, Framingham, Massachusetts), operated in positive ion reflector mode. Spectra were acquired with 50 shots of a 337 nm nitrogen laser operating at 20 Hz. Peak lists were calibrated with the trypsin autolysis fragments at m/z 842.51 and 2211.10 as internal standards.

Proteins were identified by searching the nonredundant protein database of the National Center for Biotechnology

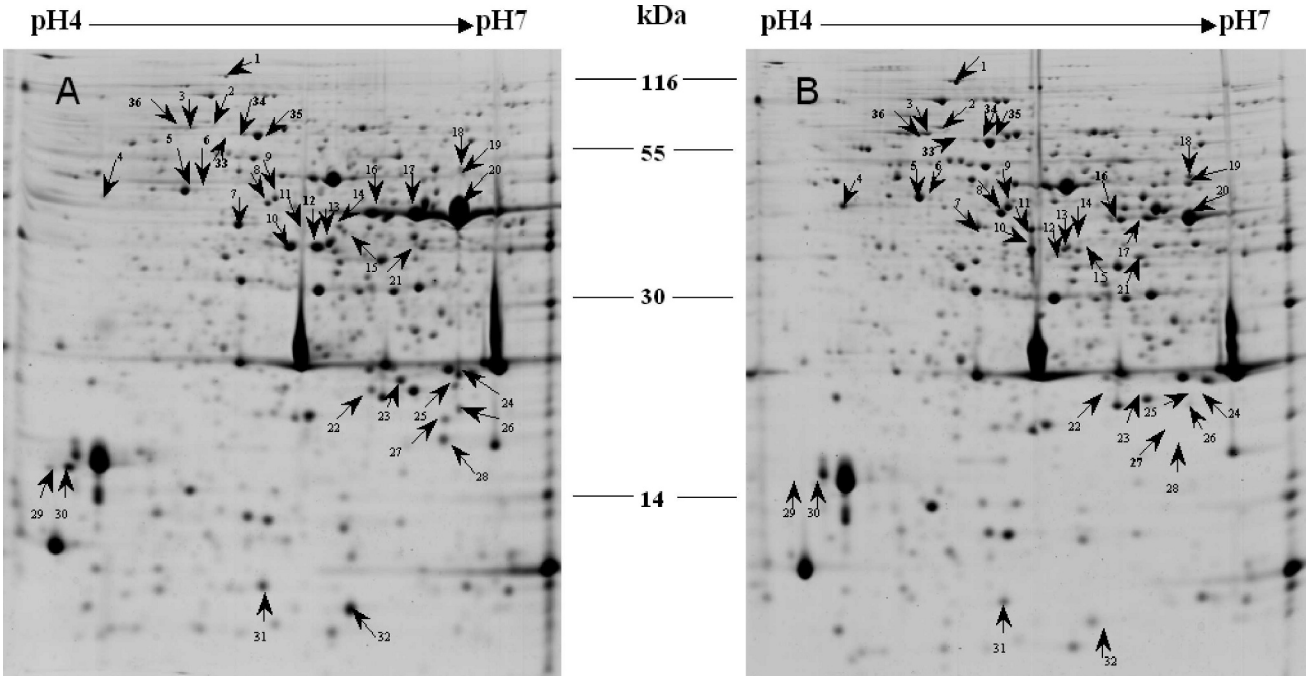

FIG. 1. Colloidal Coomassie brilliant blue G-250 stained two-dimensional, 12.5% SDS-PAGE gel images of total mycelial proteins from a 7 d old culture of *R. solani*, (AG4) isolate Rs23. Proteins (500 µg) were separated on 13 cm, pH 4–7 linear gradient immobilized pH gradient isoelectric focusing gels before second dimensional focusing. A: gel of TCA-Acetone (TA) method; B: gel of Phosphate-TCA-Acetone (PTA) method. (Numbers with arrows represent protein spots described in the manuscript and matches in TABLE I.)

Information (NCBI) with the Mascot search engine (Perkins et al 1999, http://www.matrixscience.com) and taxonomy limited to fungi. Mascot uses a probability based scoring system. These parameters were used for database searches with MALDI-TOF-MS peptide mass fingerprinting data: trypsin as digesting enzyme with one missed cleavage allowed, carbamidomethylation of cysteine as a fixed modification, monoisotopic mass, 25 ppm mass accuracy, oxidation of methionine and N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. For MALDI-TOF-MS data to qualify as a positive identification, a protein's score had to equal or exceed the minimum significant score of >68.

RESULTS

In this study we evaluated the adaptability and relative advantages of two reported protein solubilization methods for proteomic studies of *R. solani*. Emphasis has been placed on ease of extraction, total yield, migration from first dimension into the second dimension in the 2-D PAGE gels, clarity of gels, and total number, type, and intensity of protein spots.

Yield and clarity.—Following a TCA-acetone extraction procedure (TA) (Natarajan et al 2005), we obtained an *R. solani* average protein yield of 10.17 ± 1.51 mg/g mycelial fresh weight (56.46 mg/g mycelial dry weight), whereas in the phosphate extraction followed by TCA-acetone precipitation (PTA) we obtained an average protein yield of 7.78 ± 0.60 mg/g mycelial fresh weight (43.19 mg/g mycelial dry weight). It was easier to solubilize dried protein pellets after PTA than after TA. While two 10 min sonication treatments dissolved proteins from PTA, three to four sonication treatments were necessary to suspend TA pellets. Also unlike PTA a final centrifugation (14 000 rpm for 10 min) step was necessary after TA to remove insoluble debris from solubilized protein after sonication. After resuspension of precipitated proteins by sonication more debris was observed following a final centrifugation in the TA treated samples than the PTA treated samples.

2-D protein profile.—With the densitiometric tracing of Coomassie blue-stained 2-D PAGE gels (pH 4–7 [FIG. 1]) and using Image Master 2D Elite Software version 4.01 from (GE Healthcare, Piscataway, New Jersey), we were able to detect 448 ± 42 and 433.66 ± 39.67 protein spots from PTA and TA extraction methods, respectively. Overall high molecular weight protein spots were better resolved in the 2-D gel of PTA protein extracts than TA extracts (FIG. 1). However, the TA method gave rise to several lower molecular weight protein spots than the PTA method (FIG. 1). Differences in relative intensities as well as

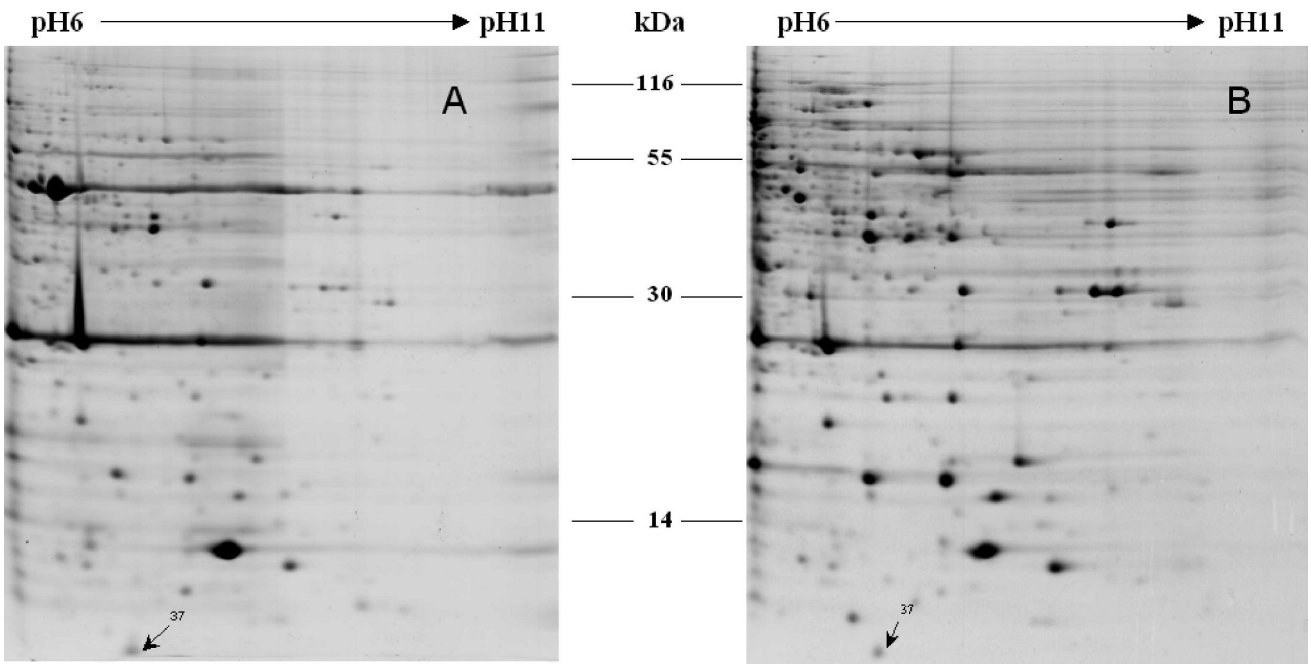

FIG. 2. Colloidal Coomassie brilliant blue G-250 stained two-dimensional, 12.5% SDS-PAGE gel images of total mycelial proteins (500 μg) from a 7 d old culture of *R. solani*, (AG4) isolate Rs23 using 13 cm, pH 6–11 linear gradient immobilized pH gradient isoelectric focusing in first dimension. A: gel of TCA-Acetone (TA) method; B: gel of Phosphate-TCA-Acetone (PTA) method. (Numbers with arrows represent protein spots described in the manuscript and matches in TABLE I.)

presence or absence of protein spots were observed among the gel-fingerprints of both the methods. For example, when we compared proteomic profiles from the same mycelial stock extracted two times using both the TA and PTA procedures, low to medium range molecular weight TA protein spots Nos. 14, 15, 16, 22, 23, 24, 25, 26, 27, 28, 29 and 30 were not visible in the PTA gel (FIG. 1). On the other hand protein spots 11 and 21 of medium molecular weight were uniquely present in the PTA gel (FIG. 1). Protein spots 10, 12, 13, 17, 20, 31 and 32 were more intense in the TA gel than in the PTA gel (FIG. 1). In contrast protein spots 4, 8, 9, 18 and 19 were more intense in the PTA gel (FIG. 1).

Protein spot identification.—Due to the absence of genomic and protein databases for *R. solani*, proteins were identified by matching peptide mass fingerprints with NCBI nonredundant protein database and with taxonomy limited to Fungi (Perkins et al 1999). Eight randomly selected matching protein spots initially were analyzed from gels of both the protein extraction methods using MALDI-TOF-MS. Of these eight spots only one (spot #5) from each gel (FIG. 1) could be identified (TABLE I). Data (TABLE I) include an assigned protein spot number, calculated isoelectric point, molecular weight, protein identity, number of peptide matched, percentage sequence coverage, MOWSE score, expected value and the NCBI database accession number of the best match and databases that yielded parallel identification. Subsequently we randomly selected 144 additional protein spots, both major and minor, from the PTA gels (FIGS. 1, 2) and processed these for MALDI-TOF-MS. In this instance only nine protein spots from pH 4–7 gel (FIG. 1; spots 1, 2, 3, 6, 7, 33, 34, 35 and 36) and one spot (FIG. 2, No. 37) from pH 6–11 gel matched previously reported fungal proteins. Among the 11 total identified protein spots, spots 3, 6, 7 and 37 were positively identified as heat shock protein 70 (gi|58264706), ATP synthase β-chain (gi|116505814), actin-1 (gi|164640790) and ubiquitin (gi|116507597) respectively (TABLE I). The other protein spots (1, 2, 5, 33, 34, 35 and 36) matched various hypothetical or predicted fungal proteins. The most probable functions (indicated in parenthesis) of the hypothetical or predicted proteins (TABLE I) were derived by comparing sequence homology with proteins of other species with basic local alignment search tool-link (BLINK) and conserved domain database (CDD) of NCBI. Spot 37 matched two proteins, ubiquitin (gi|116507597) and 4HBT (gi|149248796), respectively.

DISCUSSION

Our results showed that both TCA-acetone precipitation (TA) and phosphate solubilization of proteins

TABLE I. Proteins of *R. solani* (isolate Rs23) from 2-D PAGE, identified by MALDI-TOF-MS analysis

Spot ID	Protein identity	Theoretical pI/Mr of matched protein	Peptides matched	MOWSE score	Expected value	Sequen. coverage %	NCBI accession number
1	Hypothetical protein (*Ustilago maydis*) (Related to GroEL-like type I chaperonin)	5.41/61215	10	75	0.01	21	gil71023497
2	Hypothetical protein (*Coprinopsis cinerea*) (Related to AAA-family of ATPases)	4.91/90192	19	99	4.3e-05	20	gil116507744
3	Heat shock protein 70 (*Cryptococcus neoformans* var. *neoformans*)	4.94/69538	11	92	0.00022	19	gil58264706
5	Predicted protein (*Laccaria bicolor*) (Related to ATP synthase beta chain)	5.56/57818	14	103	1.8e-05	30	gil164650097
6	ATP synthase β-chain (*Coprinopsis cinerea*)	5.58/57948	10	85	0.0012	27	gil116505814
7	Actin-1 (*Laccaria bicolor*)	5.30/41947	8	80	0.0036	25	gil164640790
33	Predicted protein (*Laccaria bicolor*) (Related to Hsp70 chaperone)	5.49/67109	10	76	0.0081	15	gil164647040
34	Predicted protein (*Laccaria bicolor*) (Related to Hsp70 chaperone)	5.49/67109	12	82	0.0024	19	gil164647040
35	Predicted protein (*Laccaria bicolor*) (Related to Hsp70 chaperone)	5.49/67109	11	72	0.02	17	gil164647040
36	Predicted protein (*Laccaria bicolor*) (Related to Hsp70 chaperone)	5.12/71300	9	73	0.018	15	gil164650025
37	(Mixture) Ubiquitin (*Coprinopsis cinerea*)	9.96/18351	34	151	2.9e-10	12	gil116507597
	Conserved hypothetical protein (*Lodderomyces elongisporus*) (Related to 4-hydroxybenzoyl-CoA thioesterase (4HBT))						gil149248796

before TCA-acetone precipitation (PTA) methods are suitable for proteomic studies of *R. solani*. This observation is in contrast to the report of Fernández-Acero et al (2006), who observed that PTA extraction is superior to TA extraction alone for total protein yield and clarity of protein spots in 2-D gels of a filamentous ascomycetous fungus, *B. cinerea*. With TA and PTA protocols we obtained *R. solani* protein yields that are more than 28 times and seven times the reported yields for *B. cinerea* (Fernández-Acero et al 2006). The superior protein yields following both procedures in our hand may reflect minor procedural modifications, attributable to different fungal species (*B. cinerea* vs. *R. solani*) or differences in growth stages in the two instances. In conformation with our results and using a different extraction protocol Marra et al (2006) reported a protein yield of 5–10 mg per g of *R. solani* mycelial fresh weight. We also noted that the protein yields as well as quality of protein profile in the 2-D gels with both methods are comparable to a protoplast-based protein extraction method of another basidiomycetous fungus, *Tyromyces palustris* (Shimizu and Wariishi 2005).

We also compared the relative merits of the two protocols. More time was required for the PTA method. However we found it easier to solubilize proteins from dried PTA pellets than from TA pellets. Similar to the observations of Fernández-Acero et al (2006), resolution of protein spots of higher molecular weight was better in the PTA gels than in TA gels. In contrast the TA method gave rise to several lower molecular weight protein spots than the PTA method (FIG. 1). However unique protein spots were observed in the gels of each of the two protein extraction methods. In addition some protein bands were found to be more intense in the TA gels than in the PTA gels and vice versa. Obviously our findings emphasize the importance of using more than one protein extraction protocol to capture the largest protein profile in 2-D gels.

The quality of the protein spots from 2-D gels were confirmed with peptide mass fingerprinting (PMF) by

MALDI-TOF MS followed by Mascot searching. At present no public genome sequence data of *R. solani* is available. Thus we had to search for homology from protein databases of other fungi. Currently only a small number of complete and annotated genome sequences of filamentous fungi and none from family Ceratobasidiales are available in public databases. This severely limits the probability of matching *R. solani* protein PMF with annotated proteins of other fungi using MALDI-TOF MS. For example, from a total of 152 spots from gel of PTA method, only 12 proteins were identifiable. Nevertheless we were able to identify proteins from both major (No. 5) and minor (No. 6) spots (FIG. 1, TABLE I). Spots 5 and 6 match ATP synthase β-chain of *Laccaria bicolor* and *Coprinopsis cinerea* respectively. Similarly spots 33, 34, 35 (FIG. 1, TABLE I) are related to Hsp 70 chaperon. Those proteins have the same molecular weight but differ in isoelectric point; they are thus putative protein isoforms (Rodríguez-Piñeiro et al 2007). The protein (gi|116507597) in spot 36 (FIG. 1, TABLE I) has slightly lower mobility than spots 33, 34 and 35, but related to Hsp70, thus the protein (gi|116507597) in spot 36 is also a higher molecular weight isoform of Hsp 70 (Rodríguez-Piñeiro et al 2007). The two proteins in spot 37 (FIG. 2, TABLE I) ubiquitin (gi|116507597) and 4HBT (gi|149248796) respectively are unrelated but probably have occupied the same position due to similar molecular weight and isoelectric point. The detection of protein spots of high and low intensities as well protein isoforms also validates the reliability of both the protein extraction protocols. Limitations of protein identification by homology with cross-species protein sequences have been discussed (Liska and Shevchenko 2003, Habermann et al 2004, Medina et al 2004, Fernández-Acero et al 2006). Liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) has been shown to be an alternative method to identify proteins from cross-species genome sequences (Liska and Shevchenko 2003). In the future we plan to analyze *R. solani* protein spots from 2-D gels with a microspray LC-MS/MS procedure to identify more proteins that currently are not identifiable with MALDI-TOF-MS only. Also future growth in fungal genome databases and *R. solani* genome sequencing in particular (Dr Marc Cubeta pers comm and http://www.jcvi.org/cms/research/projects/wgs-rhizoctonia-solani/overview/) should enable us to more accurately identify *R. solani* proteins from 2-D gels.

It should be noted that the protocols (TA and PTA) reported in this communication are designed only for extraction of total cellular proteins of *R. solani*. Highly charged plasma membrane proteins and cell wall proteins might not have been recovered in our protein extraction methods. We are currently evaluating subcellular protein extraction protocols designed for plasma membrane- and cell wall-associated proteins (subproteomics) as well as excreted proteins (exproteome), following those developed for other filamentous fungi (Suárez et al 2005, Medina et al 2005, Asif et al 2006, Paper et al 2007). Development of protein extraction protocols, 2-D gel analysis and protein identification reported in this communication should enable us in future to develop a global database of total, developmentally and pathogenically differentially expressed proteins from *R. solani*. We anticipate that proteomic profiling of *R. solani* will advance our understanding of biology, ecology, mechanism of virulence, sensitivity to fungicides and mechanism of fungicidal actions, host-pathogen interactions and in targeting *R. solani* proteins for diagnostic purposes. In this vein we have initiated subproteomics and differential proteomic profiling of chemically induced as well as dsRNA-regulated hypovirulence of *R. solani* (Lakshman and Tavantzis 1994, Weinhold and Sinclair 1996, Lakshman et al 1998, 2006) to identify virulence-regulated proteins of the pathogen.

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